Increased Circulating Levels of 3-Nitrotyrosine Autoantibodies:
Marker for and/or Maker of Cardiovascular Disease?

Running title: Daiber et al.; 3-nitrotyrosine marker for and/or maker of cardiovascular disease

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Journal Subject Codes: [7] Chronic ischemic heart disease; [33] Other diagnostic testing; [134] Pathophysiology; [91] Oxidant stress; [95] Endothelium/vascular type/nitric oxide

Keywords: Editorials; oxidative stress; peroxynitrite; eNOS uncoupling; nitrated prostacyclin synthase; protein tyrosine nitration
3-nitrotyrosine formation is an oxidative protein modification that was first discovered in vivo in the early 1990’s by Beckman and colleagues \(^1,^2\). The biological relevance of this process was extensively investigated in the subsequent years and further facilitated by the development of 3-nitrotyrosine specific antibodies \(^3\). Protein tyrosine nitration is mainly mediated by three biochemical processes (Figure 1):

1) by peroxynitrite (ONOO\(^{−}\)) formation \(^4-^6\), the reaction product of nitric oxide (\('\text{NO}'\)) and superoxide (\('\text{O}_2^{−}'\)),

2) by a (myelo)peroxidase-catalyzed nitrogen dioxide radical (\('\text{NO}_2\)') formation from hydrogen peroxide and nitrite \(^7,^8\) and

3) by a non-specific formation of the nitrogen dioxide radical from nitric oxide in oxygenated buffers (reflecting rather artificial ex vivo conditions).

Meanwhile it is well accepted that 3-nitrotyrosine formation is associated with inflammatory conditions such as acute lung injury, rheumatoid arthritis and systemic lupus erythemathodes \(^9\), cardiovascular disease \(^10\) and neurodegenerative disorders \(^11\). More recently the group by David Harrison \(^12\) and our group \(^13\) were also able to demonstrate the crucial role of T cells and/or myelomonocytic cells such as monocytes in an angiotensin II model of hypertension in causing immune modulations, which were associated with protein tyrosine nitration processes. In addition, nitrated proteins are widely used as a clinical marker of oxidative stress (Figure 1).

**What are the pathophysiological consequences of increased tyrosine nitration of proteins?**

Protein tyrosine nitration represents a post translational modification of proteins in situations of high oxidative stress. In the setting of cardiovascular risk factors, when superoxide producing enzymes such as the NADPH oxidase \(^14\) is getting activated or the nitric oxide synthase is getting
uncoupled \textsuperscript{15} \textsuperscript{15}, \( \cdot O_2 \cdot \) will react with (and therefore metabolize) nitric oxide (\( \cdot \text{NO} \)) in an almost diffusion-controlled (this means ultra-fast) reaction \textsuperscript{16} \textsuperscript{16} that even outcompetes the detoxification of \( \cdot O_2 \cdot \) by superoxide dismutases. It is of clinical importance that this reaction not only forms the highly reactive intermediate peroxynitrite (ONOO\textsuperscript')\textsuperscript'), one of the most potent biological oxidants, but at the same time consumes the most important vasodilator principle \( \cdot \text{NO} \). The superoxide/nitric oxide reaction product peroxynitrite further impairs vascular function and generates a prothrombotic state by inhibiting prostacyclin formation via tyrosine nitration (see Supplementary Table 1 in \textsuperscript{17} \textsuperscript{17} and inactivation of prostacyclin synthase \textsuperscript{6,18} \textsuperscript{6,18} in favor of the formation of the vasoconstrictor prostaglandin endoperoxide H\(_2\) (PGH\(_2\)). More examples for direct effects of tyrosine nitration on enzymatic function and properties have been reported recently: Fibrinogen nitration has been demonstrated to increase the extent of fibrin clot formation \textsuperscript{19} \textsuperscript{19} and 3-nitrotyrosine modified Apo A-I was shown to cause a dysfunctional form of HDL with a diminished capacity to generate \( \cdot \text{NO} \) \textsuperscript{20} \textsuperscript{20}, with reduced antioxidant capacity \textsuperscript{20} \textsuperscript{20} and diminished ATP binding cassette transporter A-1 cholesterol efflux capacity from macrophages \textsuperscript{21} \textsuperscript{21} and reduced lecithin cholesterol efflux acyl transferase activity \textsuperscript{22} \textsuperscript{22}, all of which will create a prothrombotic and proatherosclerotic milieu.

For many years it is known that nitrated proteins are able to stimulate the formation of specific antibodies \textsuperscript{23} \textsuperscript{23} and that protein nitration per se, as a posttranslational modification, will further trigger immune reactions, which has been demonstrated for certain rheumatic diseases such as rheumatoid arthritis or systemic lupus erythematoses \textsuperscript{24} \textsuperscript{24}. The question whether there is an association between coronary artery disease (CAD), a disease, which is also linked with chronic inflammation and activation of the immune system, and increased levels of immunoglobulins reactive against nitrotyrosine-positive proteins has never been addressed so far.
With the present studies, Thomson et al. demonstrate for the first time that nitrated proteins exist in human atherosclerotic plaques of the carotid arteries that could serve as antigenic neoepitopes and potentially trigger the activation of the immune system\textsuperscript{17}. The authors also demonstrate that levels of circulating immunoglobulins against 3-nitrotyrosine epitopes were strikingly (ten-fold) higher in patients with CAD as compared to patients without CAD and that these levels were strongly associated with angiographic evidence of significant CAD. The specificity of their methodology was established by

- the outcompeting of the 3-nitrotyrosine binding activity of the plasma by free 3-nitrotyrosine as well as by 3-nitrotyrosine-modified proteins and peptides
- by the demonstration that a nitrated peptide reflecting an endogenous nitrated tyrosine residue identified in apo-A-I recovered from human atherosclerotic plaque, but not the native tyrosine containing peptide was able to outcompete the binding of the 3-nitrotyrosine-coupled HRP to immunoglobulins
- by the demonstration that the circulating immunoglobulins were able to recognize nitrated fibrinogen and other nitrated proteins but not the native counterparts

The mechanism for the production of immunoglobulins with selective and specific epitope recognition for 3-nitrotyrosine has been identified previously to be a consequence of termination of self-tolerance and escape of negative selection\textsuperscript{25}. The results of the present investigations just indicate that increased circulating immunoglobulins targeting protein-bound 3-nitrotyrosine are increased in patients with significant coronary artery disease.

**Conclusions and perspectives**

Although these findings per se are pretty exciting, the study delivers no answer to the question, whether this antibody formation response can be considered as a marker or a maker of CAD and
neither whether the increased antibody response may even lead to a stabilization or a destabilization of the atherosclerotic plaque and therefore to more or even less cardiovascular events?

It also brings up the chicken and egg question: do the antibody levels rise prior to overt atherosclerosis development or are they a consequence of already established atherosclerosis. It would be also important to see whether the levels are increased in patients with essential hypertension without overt cardiovascular disease and how these levels may respond in patients with CAD and hypertension to anti-atherosclerotic and antihypertensive therapy such as statins, ACE-inhibitors or angiotensin II receptor blockers. Nevertheless, the presented results demonstrating increased levels of anti-3-nitrotyrosine immunoglobulin titers in CAD patients look very promising and they may open new windows with respect to the pathophysiology, diagnosis and/or treatment of cardiovascular disease.

**Acknowledgements:** The authors thank the “Stiftung Mainzer Herz” and “Robert-Müller-Stiftung” for ongoing financial support.

**Conflict of Interest Disclosures:** None.

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Figure Legend:

Figure 1. Scheme illustrating antibody (IgG) formation against 3-nitrotyrosine-positive proteins and their involvement in the initiation and propagation of coronary artery disease (CAD). Under physiological conditions the vascular integrity is mainly guaranteed by nitric oxide (\textsuperscript{\textbullet}NO) and prostacyclin (PGI\textsubscript{2}) causing vasodilation, anti-aggregation, anti-inflammation and intact endothelial barrier function. In the presence of cardiovascular risk factor such as hypertension, chronic smoking, hypercholesterolemia and diabetes mellitus, there is increased formation of superoxide (\textsuperscript{O}_2^-) and/or increased myeloperoxidase (MPO) activity with the consequence that \textsuperscript{\textbullet}NO is consumed to form peroxynitrite (ONOO\textsuperscript{-}) or nitrogen dioxide radical (\textsuperscript{\textbullet}NO\textsubscript{2}). Peroxynitrite in turn causes tyrosine nitration of prostacyclin synthase (PGIS) with the consequence of reduced prostacyclin formation (PGI\textsubscript{2}) but also enhanced formation of the vasoconstrictor prostaglandin endoperoxide H\textsubscript{2} (PGH\textsubscript{2}). Protein nitration will also lead to a dysfunctional HDL and to enhanced fibrin clot formation. The consequences are enhanced vasoconstriction, a procoagulant state, loss of endothelial barrier function (integrity), increased expression of focal adhesion proteins, white blood cell (WBC) adhesion and infiltration and subsequently increased protein tyrosine nitration (3NT). Antibodies formed against 3-nitrotyrosine-positive proteins may be used as a new biomarker for the detection of the activity of the atherosclerotic process and may also represent a new target for cardiovascular disease treatment (e.g. by using antioxidants as suppressors of protein tyrosine nitration).
\[ \text{NO} + \text{PGI}_2 \]

- Vasodilation
- Anti-thrombotic
- Endothelial integrity

\[ \text{NO}_x + \text{PGH}_2 \]

- Consumption of \('NO\), inactivation of \(\text{PGI}_2\)
- Vasoconstriction, prothrombotic state
- Loss of endothelial integrity
- WBC adhesion and infiltration

Cardiovascular risk factors:
- Smoking
- Hypercholesterolemia
- Hypertension
- Diabetes mellitus

\[ \text{O}_2 \rightarrow \text{ONOO}^- \]

\[ \text{MPO/H}_2\text{O}_2 \rightarrow \text{NO}_2 \]

- Further recruitment of WBC, increased \('O_2^-\)
- Formation and MPO activity

Detection of specific antibodies for diagnosis

IgG formation
- Immune response
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Circulation. published online October 18, 2012; Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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http://circ.ahajournals.org/content/early/2012/10/17/CIRCULATIONAHA.112.143214

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